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Engineering and Analysis of a *Saccharomyces cerevisiae* Strain That Uses Formaldehyde as an Auxiliary Substrate[∇]

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We demonstrated that formaldehyde can be efficiently coutilized by an engineered *Saccharomyces cerevisiae* strain that expresses *Hansenula polymorpha* genes encoding formaldehyde dehydrogenase (*FLD1*) and formate dehydrogenase (*FMD*), in contrast to wild-type strains. Initial chemostat experiments showed that the engineered strain coutilized formaldehyde with glucose, but these mixed-substrate cultures failed to reach steady-state conditions and did not exhibit an increased biomass yield on glucose. Subsequent transcriptome analyses of chemostat cultures of the engineered strain, grown on glucose-formaldehyde mixtures, indicated that the presence of formaldehyde in the feed caused biotin limitations. Further transcriptome analysis demonstrated that this biotin inactivation was prevented by using separate formaldehyde and vitamin feeds. Using this approach, steady-state glucose-limited chemostat cultures were obtained that coutilized glucose and formaldehyde. Coutilization of formaldehyde under these conditions resulted in an enhanced biomass yield of the glucose-limited cultures. The biomass yield was quantitatively consistent with the use of formaldehyde as an auxiliary substrate that generates NADH and subsequently, via oxidative phosphorylation, ATP. On an electron pair basis, the biomass yield increase observed with formaldehyde was larger than that observed previously for formate, which is tentatively explained by different modes of formate and formaldehyde transport in *S. cerevisiae*.

In industrial biotechnology, the carbon feedstock is usually a sugar or sugar-rich substrate such as molasses, which often represents an important cost factor. In many processes, a large portion of the sugar feed is not converted into product but is instead dissimilated to provide free energy (e.g., in the form of ATP equivalents or proton motive force) and/or reducing power [in the form of NAD(P)H]. The use of mixed substrates consisting of a sugar and an additional, inexpensive source of reducing power and/or free energy represents an interesting approach to reduce the costs of industrial fermentation. Ideally, this practice should lead to a situation in which the sugar is used exclusively as a carbon source for biomass and/or product formation. The validity of this “auxiliary substrate” concept has been demonstrated in many academic studies using compounds such as formate or thiosulfate, whose oxidation can provide free energy but which cannot be used as carbon sources (2, 7, 26).

Methanol is a low-cost chemical which can be derived from fossil sources or from biomass (via syngas, a CO- and H₂-containing gas mixture generated by gasification of organic matter) (9, 17) and is an interesting candidate for the role of “auxiliary substrate” in industrial biotechnology. However, only a few industrial microorganisms are capable of utilizing methanol, including several yeast species that can grow on

methanol as the sole carbon and energy source (35, 42, 46). In these methylotrophic yeasts, like *Hansenula polymorpha* and *Pichia pastoris*, methanol is first oxidized to formaldehyde by an alcohol oxidase. The formaldehyde produced is then either assimilated by the xylulose-5-phosphate cycle or dissimilated via formate to carbon dioxide and water by two NAD⁺-dependent enzymes, formaldehyde dehydrogenase (Fld) and formate dehydrogenase (Fmd) (for a recent review see reference 42).

The yeast *Saccharomyces cerevisiae* can be used as a metabolic engineering platform for the large-scale production of a broad range of products as diverse as C₄-dicarboxylic acids (29), hydrocortisone (39), and artemisinic acid (34). In contrast to methylotrophic yeast, *S. cerevisiae* is unable to utilize methanol (12, 43). While *S. cerevisiae* lacks an alcohol oxidase, it does contain genes encoding Fld and Fmd (16, 30). It has recently been demonstrated that formate can be used as an auxiliary substrate for improving biomass yields from aerobic cultures (30) and glycerol yields from both aerobic and anaerobic glucose-grown cultures of *S. cerevisiae* (14, 16). Analysis of the energy efficiency of formate coutilization showed a lower-than-anticipated contribution to biomass yields, which was tentatively attributed to the energy costs of formate uptake (30).

The use of formaldehyde as an auxiliary substrate is an essential step toward the ultimate goal of introducing a linear oxidation pathway for methanol in *S. cerevisiae*. Moreover, analysis of the energetics of formaldehyde oxidation is relevant for testing previous assumptions about the energetics of formate transport (30). Preliminary attempts in our laboratories to feed formaldehyde to glucose-limited chemostat cultures of wild-type *S. cerevisiae* strains consistently led to a washout of

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the cultures, due possibly to formaldehyde toxicity (8, 49) and an insufficient endogenous capacity for formaldehyde oxidation (J. T. Pronk and J. M. A. Geertman, unpublished observations).

The overexpression of the endogenous *S. cerevisiae* gene encoding formaldehyde dehydrogenase (*SFA1*) was previously shown to result in enhanced formaldehyde resistance, presumably via formaldehyde oxidation (41). The aim of the present study was to investigate whether high-level expression of heterologous Fld and of Fmd would enable the use of formaldehyde as an auxiliary substrate by aerobic, glucose-limited chemostat cultures of *S. cerevisiae*. To this end, we constructed *S. cerevisiae* strains that expressed the structural genes for Fld and Fmd from the methylotrophic yeast *H. polymorpha*. Subsequently, biomass yields and metabolic fluxes were analyzed in aerobic chemostat cultures of an engineered strain, grown on glucose-formaldehyde mixtures. Transcriptome analysis was applied to investigate and resolve problems related to the stability of the chemostat cultures.

MATERIALS AND METHODS

Microorganisms and growth conditions. The *S. cerevisiae* strains used in this study are as follows: CEN.PK113-7D (*MATa URA3 HIS3 LEU2 TRP1 MAL2-8C SUC2*), CEN.PK113-3C (*MATa URA3 HIS3 LEU2 trp1-289 MAL2-8C SUC2*), CEN.PK113-5D (*MATa ura3-52 HIS3 LEU2 TRP1 MAL2-8C SUC2*), and CEN.PK113-11A (*MATa URA3 his3-Δ1 LEU2 trp1-289 MAL2-8C SUC2*). These strains were provided by P. Kötter (Euroscarf, Frankfurt, Germany) and originate from the CEN.PK family, which was previously identified as a suitable background for combined genetic and physiological studies (44). Derivatives were obtained by transformation with a plasmid-prototrophic wild-type strain, as follows: the CEN.PK113-5D strain with the pRS316 plasmid (38); a *SFA1*-overexpressing strain, CEN.PK113-5D with plasmid pRUL129 (41); a *FLD1*-expressing strain, CEN.PK113-3C with pSUM2T-P_{TDH3}*FLD1*; and a *FLD1/FMD*-expressing strain, CEN.PK113-11A with pSUM2T-P_{TDH3}*FLD1* and pSUM2H-P_{TPH1}*FMD*.

S. cerevisiae strains were grown to stationary phase at 30°C in shake flasks on synthetic medium set at pH 6.0 and supplemented with vitamins and 2% (wt/vol) glucose (47). Stock cultures (2-ml cell aliquots containing 20% [vol/vol] glycerol) were stored at -80°C and used for inoculation of the batch experiments or precultures for the chemostat cultures. For transformations and initial strain analyses, cells were grown in YND medium containing 0.67% yeast nitrogen base (Difco, Sparks, MD) and 1% glucose. For growth on plates, 2% agar was added to the medium.

Hansenula polymorpha CBS4732 (27) was cultivated at 37°C in YPD media containing 1% yeast extract, 1% peptone, and 1% glucose. For plasmid construction, selection, and propagation, *Escherichia coli* DH5α and DB3.1 (Invitrogen, Breda, The Netherlands) were used and cultivated as described previously (36).

Chemostat cultivation. Aerobic chemostat cultivation was performed at 30°C in 2.0-liter laboratory fermentors (Applikon, Schiedam, The Netherlands) with a stirrer speed of 800 rpm. The working volume was kept at 1 liter by a Masterflex peristaltic effluent pump (Barrington, IL) coupled to an electric level sensor. The pH was kept at 5.0 by using an Applikon ADI 1030 biocontroller via the automatic addition of 2.0 M potassium hydroxide (15). Cultures were sparged with air at a flow rate of 0.5 liter min⁻¹ using a Brooks model 5876 mass flow controller. The dissolved oxygen tension was monitored continuously with an oxygen electrode (Ingold model 34.100.3002; Mettler, Utrecht, The Netherlands) and remained above 50% of the air saturation in all chemostat cultures. The dilution rate (in steady-state cultures equal to the specific growth rate) was set to 0.10 h⁻¹.

Synthetic medium was prepared as described previously (47) with glucose (7.5 g liter⁻¹) as the sole carbon source. Filter-sterilized vitamins were either added directly to the medium or added from a separate reservoir. Formaldehyde was prepared by hydrolyzing para-formaldehyde in 15 mM ammonium hydroxide for a 20-min incubation at 100°C. The solution was aseptically added to the medium reservoir at various concentrations, as indicated in Results. When formaldehyde was cofed, *S. cerevisiae* was first cultivated in a batch culture on glucose alone, followed by 24-h chemostat cultivation with 30 mM formaldehyde in the medium

vessel, after which the medium was switched to the final concentration of formaldehyde until a steady-state culture was established. Steady state was defined as the situation in which at least 5 volume changes had passed since the last change in culture parameters and in which the biomass concentration, as well as all other specific production or consumption rates, had remained constant (<2% variation) for at least 2 volume changes. Steady-state cultures did not exhibit detectable metabolic oscillations and were routinely checked for purity by phase-contrast microscopy.

DNA procedures. Standard recombinant DNA manipulations were performed as described previously (36). Transformation of *S. cerevisiae* cells was performed according to the method described by Knop et al. (22). Chromosomal DNA of YPD-grown *H. polymorpha* cells was extracted using the method described by Sherman et al. (37), but it included an additional protein precipitation step using 5 M sodium chloride prior to DNA precipitation. DNA-modifying enzymes were used as recommended by suppliers Roche (Almere, The Netherlands) and Fermentas (St. Leon-Rot, Germany). *Pyrococcus woesei* (*Pwo*) polymerase (Roche) was used for PCRs. Oligonucleotides were synthesized by Biolegio (Nijmegen, The Netherlands); their sequences are available on request. DNA sequencing was performed by BaseClear and ServiceXS (Leiden, The Netherlands).

Plasmid constructions. To enable the construction of vectors hosting the expression of the *H. polymorpha* *FLD1* (*HpFLD1*) (3) and *HpFMD* genes (19) under the control of the *S. cerevisiae* *TDH3* or *TPH1* promoters, an Invitrogen Multisite Gateway three-fragment recombinational cloning technology was applied and carried out as recommended by the supplier (18). *E. coli* strain DB3.1 (Invitrogen, Breda, The Netherlands) was used for the construction and/or propagation of the gateway destination vector pDEST_R4-R3 and its derivatives and donor vectors pDONR_P4-P1R, pDONR_221, and pDONR_P2R-P3 (Invitrogen, Breda, The Netherlands). The correct sequence of each vector was confirmed by DNA sequencing.

To accommodate the high and stable expression of *HpFLD1* or *HpFMD* in *S. cerevisiae*, we adapted the pDEST_R4-R3 destination vector to a high-copy-number and autonomously replicating vector by introducing the 2-μm origin of replication and a selectable marker, i.e., *HIS3* or *TRP1*. A 2.9-kb fragment containing the 2-μm origin of replication and the *TRP1* marker was amplified by PCR using pESC-TRP (Stratagene, Amsterdam, The Netherlands) as the template and the primers *sum021* and *sum022*. After this fragment was digested with AatII-NdeI, it was cloned into an AatII-NdeI-digested pDEST_R4-R3 vector, yielding pSUM2T. Similarly, a 2.8-kb fragment containing the 2-μm origin of replication and the *HIS3* marker was amplified by PCR using pRS423 (10) as the template and primers *sum043* and *sum044*. These last primers introduce, next to NarI and AatII sites, four additional restriction sites (i.e., NarI, XhoI and SacI; and AatII, SpeI and SphI); hereby, after cloning into pDEST_R4-R3, the resulting vector is more accessible for future modifications. The amplified fragment was digested with NarI-AatII and ligated into a NarI-AatII-digested pDEST_R4-R3, yielding pSUM2H.

Two promoter entry vectors, derived from the recombination with pDONR_P4-P1R, were constructed, containing (i) the *TDH3* promoter (previously named *GPD*) by the amplification of a 0.7-kb fragment from p416GPD (28) by PCR, using primers *sum001* and *sum003*, yielding pSUM1; and (ii) the *TPH1* promoter by the amplification of a 0.9-kb fragment from YEplac181-P_{TPH1}-*TCYC1* (31) by PCR using the primers *sum006* and *sum007*, yielding pSUM4. A terminator entry vector, derived from the recombination with pDONR_P2R-P3, was constructed, containing the terminator sequence of *CYC1* by the amplification of a 0.15-kb fragment containing the terminator sequence of *CYC1* that was amplified by PCR from pESC-TRP, using the primers *sum016* and *sum015*, recombined into the donor vector, yielding pSUM91.

Gene entry vectors hosting *HpFLD1* and *HpFMD* were derived from the recombination into pDONR_221. The 1.1-kb *HpFLD1* gene was amplified by PCR using the primer pairs *sum008* and *sum010*, using *H. polymorpha* CBS4732 chromosomal DNA as the template, which was subsequently applied to in vitro recombination into pDONR_221, yielding pSUM10. The 1.1-kb *HpFMD* gene was amplified by PCR using the primer pairs *sum011* and *sum013*, using chromosomal *H. polymorpha* CBS4732 DNA as the template, subsequently used for the in vitro-recombination into pDONR_221, yielding pSUM12.

Finally, the entry vectors pSUM1 (*P_{TDH3}*), pSUM10 (*FLD1*), and pSUM91 (*TCYC1*), along with the destination vector pSUM2T, were applied to construct the expression vector pSUM2T-P_{TDH3}*FLD1*. To construct pSUM2H-P_{TPH1}*FMD*, the entry vectors pSUM4 (*P_{TPH1}*), pSUM12 (*FMD*), and pSUM91 (*TCYC1*) were recombined in the destination vector pSUM2H. During LR recombination reactions, occasionally the *attR4* and *attR3* sites of pSUM2H and pSUM2T displayed recombination to each other, leading to "empty" vectors that have lost the *ccdB* and *Cm^r* cassettes (confirmed by DNA sequencing). These vectors were used as controls.

Analytical procedures. Biomass dry mass determination, substrate and metabolite analyses, off-gas analysis, preparation of cell extract, and determination of chemostat culture protein samples were performed as described previously (15). Formate concentrations were confirmed by a colorimetric method (24). Fld and Fmd enzyme activities were assayed in cell extracts at 30°C, according to the method described by van Dijken et al. (45). Protein concentrations were determined by the Lowry method (25) or by using a Bio-Rad assay kit (Bio-Rad GmbH, Munich, Germany), using bovine serum albumin as a standard.

Microarray analysis. Sampling of the chemostat-grown cells from aerobic glucose-limited chemostats with or without cofeeding of formaldehyde, probe preparation, and hybridization to Affymetrix GeneChip microarrays were performed as described previously (21). The results for each growth condition were derived from duplicate independent experiments when biotin and formaldehyde were present in the same medium reservoir and from three independently cultured replicates for all other conditions.

Data acquisition and analysis. Acquisition and quantification of array images and data filtering were performed using Affymetrix GeneChip operating software version 2.1. Before comparisons were made, all arrays (using ".CHP" file extensions) were globally scaled to a target value of 150, using the average signal from all gene features. To eliminate insignificant variations, genes with values below 12 were set to 12 as described in reference 32. From the 9,335 transcript features on the YG-S98 arrays, a filter was applied to extract 6,383 yeast open reading frames, of which there were 6,084 different genes. This discrepancy was because several genes were represented more than once when suboptimal probe sets were used in the array design. To represent the variation in triplicate measurements, the coefficient of variation (standard deviation divided by the mean) was calculated as described previously (5). For additional statistical analyses, Microsoft Excel running the significance analysis of microarrays (SAM; version 1.12) add-in was used for pairwise comparisons (40). Genes were considered to be changed in expression if they were called significantly changed from each other by at least twofold, using SAM (with an expected median false discovery rate of 1%). The statistical assessment of overrepresentation of Munich Information Center for Protein Sequences (MIPS) categories among sets of significantly changed transcripts was achieved using a Fisher exact test as previously described (21, 23).

Microarray data accession number. The microarray data have been deposited in the Genome Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) (4) under the accession series number GSE8902.

RESULTS

Expression of HpFLD1 in *S. cerevisiae*. Since methylotrophic yeasts such as *H. polymorpha* have an efficient, high-capacity pathway for formaldehyde dissimilation, we introduced the HpFLD1 (3) and HpFMD (41) genes that are involved in this pathway in *S. cerevisiae*. The HpFLD1 gene was placed under the control of the strong glyceraldehyde-3-phosphate dehydrogenase promoter (*TDH3*) (vector pSUM2T-*P_{TDH3}*-FLD1). FLD1-containing transformants and reference cells harboring an empty vector were grown in batch cultures on glucose-containing medium (YND) and used for enzyme activity measurements. In cell extracts of the empty vector strain, Fld activities were invariably low (0.1 ± 0.0 U mg⁻¹ protein). These activities were strongly enhanced in the FLD1-expressing strain (4.5 ± 0.1 U mg⁻¹ protein), indicating that the *H. polymorpha* gene was functionally expressed in the heterologous host.

Subsequently, we investigated whether FLD1-expressing cells acquired resistance to formaldehyde, as was previously shown for SFA1-overexpressing *S. cerevisiae* cells (41). As shown in Fig. 1, transformants harboring the FLD1 expression vector were capable of growing on YND plates in the presence of enhanced formaldehyde concentrations (up to 30 mM) relative to that of the empty vector control strain (up to 2 mM). In addition, the FLD1-expressing *S. cerevisiae* cells were resistant to higher formaldehyde concentrations than the cells that overexpressed the native *S. cerevisiae* Fld gene SFA1 under the control of the *TDH3* promoter (15 to 20 mM) (Fig. 1).

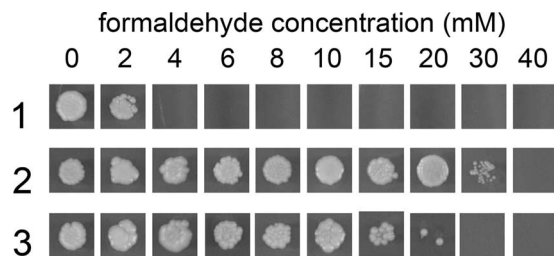


FIG. 1. Growth of *S. cerevisiae* strains on YND agar plates containing formaldehyde (at 0, 2, 4, 6, 8, 10, 15, 20, 30, and 40 mM) after incubation at 30°C for 4 days. Row 1, wild-type strain transformed with the empty vector pRS316; row 2, FLD1-expressing strain; row 3, SFA1-overexpressing strain. Cells of each strain were spotted with 10 \times diluted YND-grown overnight cultures.

The HpFMD gene was also introduced in the FLD1-expressing strain (resulting in the FLD1/FMD strain). HpFMD was placed under the control of the strong triosephosphate isomerase (*TPI1*) promoter. Enzyme activity measurements using glucose-grown FLD1/FMD cells (in batch cultures on YND) revealed an Fmd activity of 0.1 ± 0.0 U mg⁻¹ protein, whereas in glucose-grown reference cells containing the empty vector, the Fmd activity was below the limit of detection. The Fld activities in the FLD1/FMD strain were similar to those in the strain expressing FLD1 alone (4.4 ± 0.2 U mg⁻¹ protein versus 4.5 ± 0.1 U mg⁻¹ protein, respectively).

The addition of formaldehyde to the feed results in biotin depletion. The addition of formaldehyde to the feed of aerobic glucose-limited pilot experiments with the engineered *S. cerevisiae* FLD1/FMD strain did not result in steady-state cultures. Although formaldehyde concentrations remained below the detection limit (1 mM), residual formate concentrations fluctuated, whereas the biomass concentrations tended to decline over several days of cultivation. Also, the biomass yield on glucose did not exceed that of the reference cultures grown on glucose only (data not shown). To identify potential bottlenecks and to understand this undesired response of *S. cerevisiae* to formaldehyde as cosubstrate, these unstable cultures were harvested for genome-wide expression profiling by DNA microarrays after 1 week of cultivation. The transcript data obtained from duplicate cultures revealed that the complete set of biotin biosynthetic genes (i.e., *BIO2*, *BIO3*, *BIO4*, and *BIO5*), as well as the biotin transporter *VTH1*, were significantly upregulated (ranging from +5.7-fold to +24.7-fold) when formaldehyde was added to the feed vessel (Fig. 2B). Coordinated upregulation of biosynthetic genes is often indicative of a nutrient limitation (33).

In these mixed substrate cultures, a biotin limitation may have been caused by a chemical reaction between formaldehyde and biotin in the medium reservoir (6). Based on this initial transcriptome analysis, a new experimental setup was designed in which the vitamin mixture (containing biotin) and formaldehyde were fed to the chemostat cultures from separate reservoirs to avoid prolonged interaction between the two components. With formaldehyde fed from a separate vessel, steady states were readily achieved. Under these new experimental conditions, 55 genes were significantly upregulated, and 148 genes were downregulated in the engineered strain grown on glucose-formaldehyde mixtures relative to the transcrip-

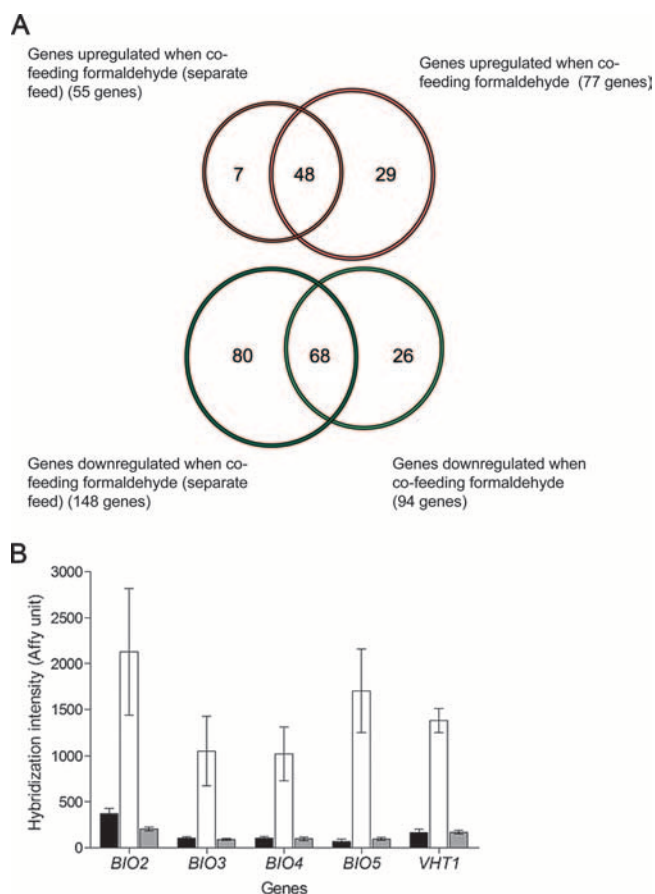


FIG. 2. Effect of the fermentation protocol on genome-wide gene expression profiling. (A) Venn diagrams compare the number of differentially upregulated (red) or downregulated (green) genes obtained when formaldehyde was added directly to the medium vessel (right side) and when formaldehyde and vitamins were fed separately (left side). (B) Hybridization intensity of the biotin biosynthetic and uptake genes. Filled bars, wild-type strain, glucose-limited chemostat cultivated; open bars, *FLD1/FMD*-expressing strain formaldehyde-glucose-limited chemostat cultivated; gray bars, *FLD1/FMD*-expressing strain, formaldehyde was separately fed glucose-limited chemostat cultivated.

tome of the reference strain CEN.PK113-7D grown in glucose-limited chemostat cultures. Importantly, by using separate biotin and formaldehyde feeds, the expression of the biotin biosynthetic genes was no longer upregulated compared to that of the reference conditions without formaldehyde. Interestingly, 87% (48 of 55 genes) of the other genes that were upregulated in the original experimental design were upregulated again, and 46% (68 of 148 genes) of the downregulated genes were also downregulated in the novel experimental setup. This indicates that to a large extent, these genes responded primarily to the presence of formaldehyde (Fig. 2A). Analysis of the functional categories of the differentially expressed genes identified significant overrepresentation of several MIPS functional categories among the formaldehyde-responsive genes. Out of 55 genes that were upregulated in formaldehyde-coultivating cultures, genes belonging to the MIPS functional categories of detoxification (7 genes) and oxidative stress (4 genes) were significantly enriched (Fig. 3).

Coconsumption of formaldehyde in glucose-limited chemostat cultures. The effects of the addition of formaldehyde to glucose-limited chemostat cultures of the *FLD1/FMD*-expressing *S. cerevisiae* cultures on biomass yield and enzyme activities were studied in detail. At a constant glucose concentration in the feed of 7.5 g liter⁻¹, steady-state cultures were obtained at formaldehyde concentrations in the feed of up to 55 mM (representing a molar formaldehyde-to-glucose ratio of 1.32 mol mol⁻¹) (Fig. 4A). In these cultures, the residual concentrations of glucose and formaldehyde were below the detection level of the routine analytical tools (0.5 mM and 1.0 mM, respectively), and calculated carbon recoveries were 104.3% ± 2.6%. A further increase in formaldehyde concentration (above 55 mM) resulted in fluctuations in the culture parameters, and no steady states were reached (data not shown).

When the formaldehyde concentrations in the feed were increased from 0 to 55 mM, the original specific glucose consumption rate (1.15 mmol g⁻¹ h⁻¹) decreased to 1.00 mmol g⁻¹ h⁻¹, together with a specific formaldehyde consumption rate of 1.37 mmol g⁻¹ h⁻¹. The addition of 55 mM formaldehyde resulted in a 12% increase in biomass yield on glucose. With increasing formaldehyde concentrations, low concentrations of formate were detected in culture supernatants, which reached approximately 1 mM when 55 mM formaldehyde was added (Fig. 4A).

Enzyme activity measurements (Fig. 4B) revealed that the Fld activities ranged between 8.4 and 13.4 U mg⁻¹ protein, indicative of the efficient expression of Hp*FLD1* (Fig. 4B). Formate dehydrogenase activities increased from 0.05 U mg⁻¹ protein in the absence of formaldehyde to ca. 0.27 U mg⁻¹ protein at the highest formaldehyde concentration (55 mM) in the feed.

DISCUSSION

In this study we demonstrate that an engineered *S. cerevisiae* strain can coutilize glucose and formaldehyde in steady-state glucose-limited chemostat cultures. Coutilization resulted in an enhanced biomass yield of the glucose-limited cultures, which was quantitatively consistent with the use of formaldehyde as an auxiliary substrate.

Transcriptome analysis is a powerful method for detecting bottlenecks in formaldehyde cofeeding. Formaldehyde was efficiently coutilized by an engineered *S. cerevisiae* strain that expresses *H. polymorpha* genes encoding Fld and Fmd (3, 19). Transcriptome analysis played a key role in identifying a biotin limitation in the initial fermentation experiments with formaldehyde coutilization by chemostat cultures. The strong biotin limitation response observed when formaldehyde and biotin were present in the same medium reservoir indicates that the inactivation of biotin by formaldehyde may occur at much lower concentrations and under milder conditions than was previously reported (6). A simple adaptation of the experimental setup, in which the formaldehyde and the vitamin mixture were added separately to the chemostat culture, enabled stable coutilization of formaldehyde and glucose by the engineered strains.

In this study, transcriptome analysis not only enabled the redesign of the experiment, thereby eliminating the biotin limitation, but also revealed interesting information about the

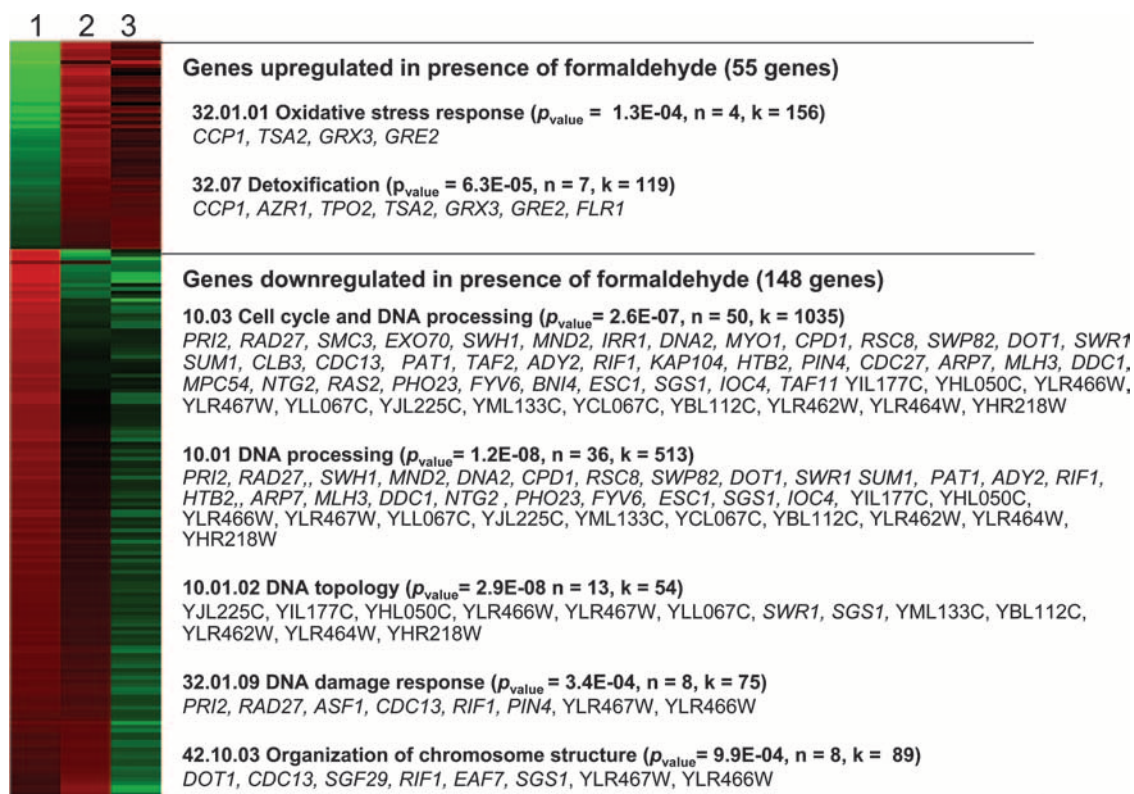


FIG. 3. Heat map showing the transcript profiles of 203 genes that showed statistically different transcript levels in cultures of an engineered *FLD1/FMD*-expressing *S. cerevisiae* strain (separate formaldehyde feeding) compared to those of glucose-limited cultures of a reference strain. The genes indicated in the figure belong to the enriched MIPS categories found in the up- and downregulated gene clusters based on Fisher's exact test ($P < 0.001$). Lane 1, reference strain grown in aerobic, glucose-limited chemostat cultures; lane 2, *FLD1/FMD*-expressing strain grown on formaldehyde-glucose mixture in aerobic carbon- and energy-limited chemostat, with the formaldehyde and vitamin mixtures in the same medium reservoir; lane 3, *FLD1/FMD*-expressing strain grown on a formaldehyde-glucose mixture in aerobic carbon- and energy-limited chemostat, with formaldehyde and vitamin mixtures fed from different medium reservoirs. The P value represents the significance of overrepresentation of MIPS categories among sets of significantly changed transcripts according to Fischer's exact test (23); n is the number of genes belonging to a MIPS category in the set of differentially expressed genes, and k is the number of genes that belong to the same category genome wide. Biotin-responsive genes, which were upregulated only when formaldehyde and biotin were present in the same medium feed (Fig. 2B), are not included in this figure.

transcriptional responses of the engineered *S. cerevisiae* strain to formaldehyde with the improved fermentation setup. Seven genes belonging to the MIPS functional categories of detoxification and oxidative stress (Fig. 3) showed increased transcript levels in formaldehyde-coultivating cultures of the engineered strain. Four of these genes, *CCP1*, *TSA2*, *GRX3*, and *GRE2*, are involved in detoxification and the response to oxidative stress. Formaldehyde reacts with reduced glutathione to form *S*-hydroxymethyl glutathione, which forms the actual substrate for *H. polymorpha* Fld (45). In *S. cerevisiae*, the formation of *S*-hydroxymethyl glutathione may reduce the level of reduced intracellular glutathione and hence affect the oxidative state of the cells. Three additional genes in the MIPS detoxification category that were upregulated, *AZR1*, *FLR1*, and *TPO2*, encode transporters. Their role might be related to formaldehyde detoxification by export. *Azr1* and *Flr1* are multidrug resistance proteins belonging to the major facilitator superfamily (MFS-MDR) and have been implicated in the export of various compounds (1, 20). *TPO2* is required for rapid adaptation to weak acid food preservatives such as propionic or acetic acid (13) and may be upregulated as a response to formate formation (Fig. 4A). The *S. cerevisiae* formate de-

hydrogenase genes *FDH1* and *FDH2* were both significantly upregulated, most probably as a result of intracellular formate formation. This is in line with earlier data that showed that *FDH1* and *FDH2* are upregulated upon the addition of formate to glucose-limited *S. cerevisiae* cultures (30). Furthermore, as shown in Fig. 4B, *Fmd* activities increased in response to higher formaldehyde-to-glucose ratios. Remarkably, the expression of the endogenous *SFA1* gene in the *FLD1/FMD*-expressing strain was not significantly upregulated by formaldehyde. This contrasts with the situation for wild-type cells (16, 49), suggesting that *H. polymorpha* Fld efficiently removes intracellular formaldehyde in the engineered strain. Furthermore, the expression of *YJL068C*, encoding an intracellular esterase proposed to function as an *S*-formylglutathione hydrolase in formaldehyde dissimilation (11), was also not formaldehyde upregulated, suggesting that the gene is constitutively expressed or that its protein product has a limited role in formaldehyde detoxification (data not shown).

Formaldehyde as an auxiliary substrate for engineered *S. cerevisiae*. We showed that *S. cerevisiae* can be engineered to use formaldehyde as an auxiliary substrate in glucose-limited chemostat cultures. In such cultures, the NADH produced by

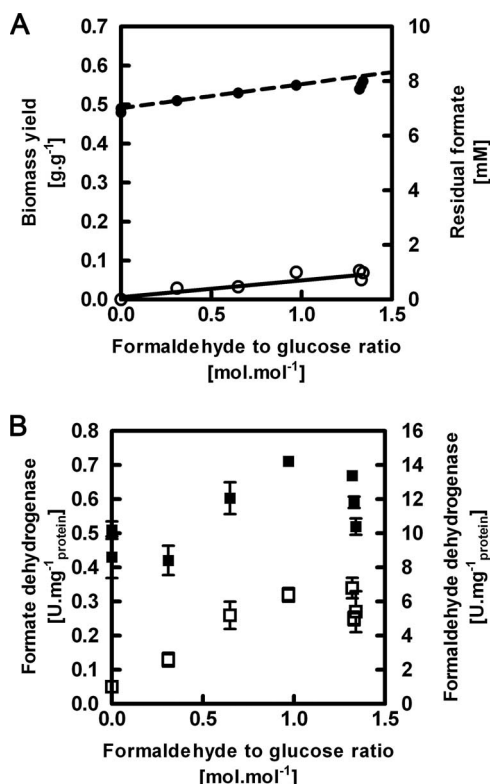


FIG. 4. Aerobic, carbon- and energy-limited chemostat cultivation of an engineered *S. cerevisiae* strain expressing the HpFLD1 and FMD genes, grown at different molar ratios of formaldehyde and glucose. (A) Biomass yield (Y_{sx}) (filled circles) and residual formate concentration in culture supernatants (open circles) are shown. The dashed line represents a theoretical prediction of the biomass yield on formaldehyde-glucose mixtures, based on the assumption that redox equivalents from glucose and formaldehyde share the same in vivo P/O ratio of 1.0 (48). (B) Specific activities of Fmd (open squares) and Fld (closed squares) as a function of the molar ratio of formaldehyde and glucose in the feed of chemostat cultures.

the dissimilation of formaldehyde and subsequently the ATP generated via oxidative phosphorylation can replace ATP that, during growth on glucose alone, would have to be derived from the respiratory dissimilation of glucose. At an effective in vivo P/O ratio (ATP formed per electron pair in oxidative phosphorylation) of 1.0 (48), complete respiratory dissimilation of glucose can yield 16 ATP, including 4 ATP equivalents derived from substrate-level phosphorylation reactions in glycolysis and the tricarboxylic acid cycle. Similarly, oxidation of the 2 NADH formed in the oxidation of formaldehyde to CO_2 can yield 2 ATP. Theoretically, the oxidation of 1 mol of formaldehyde should then replace $2/16 = 0.125$ mol glucose. Based on the biomass yield on glucose in the absence of formaldehyde (i.e., 0.491 g^{-1}), the relationship between the apparent biomass yield on glucose (Y_{sx}) and the molar ratio of formaldehyde to glucose, F , is then predicted to follow the linear equation $Y_{sx} = 0.491 + (0.125 \times 0.491)F = 0.491 + 0.0614F$. The experimental data presented in Fig. 4A are in good agreement with this prediction.

Overkamp et al. (30) demonstrated that cointilization of formate by glucose-limited chemostat cultures (up to a molar ratio of 5.4, which corresponds to a formate concentration of

228 mM) also led to increased biomass yields. However, the experimentally determined biomass yields of the mixed substrate chemostat cultures were lower than the theoretical values. It was suggested that a formate-dependent, energy-coupled process, like formate uptake, is responsible for the low energetic efficiency of formate oxidation (30). Our current data are in line with this suggestion, since formaldehyde utilization in the FLD1/FMD strain generates formate in the cytosol, excluding possible uptake and/or energy requirements prior to oxidation of this compound.

The theoretical maximum biomass yield on glucose in the presence of an auxiliary substrate is reached when glucose is used only for assimilation and the provision of NADPH, whereas all the additional ATP required for biosynthesis is generated from dissimilation of the auxiliary substrate. For yeast, assuming the free diffusion of formaldehyde and a P/O ratio of 1.0, the theoretical maximum yield on glucose would amount to 0.68 g g^{-1} (7). According to the prediction discussed above, this theoretical maximum would be reached at a formaldehyde-to-glucose molar ratio of 3.1, which was not reached in our experiments, probably due to the insufficient in vivo capacity of formaldehyde dissimilation. Thus, while we demonstrated that the strategy chosen for enabling formaldehyde dissimilation is stoichiometrically valid, there is room remaining for further improvement of the kinetics of this process with engineered *S. cerevisiae* strains. The transcriptome data discussed in the preceding paragraph suggest that the formation of S-hydroxymethyl glutathione may be a relevant reaction in this respect.

The ability of the FLD1/FMD-engineered *S. cerevisiae* strain to efficiently utilize formaldehyde as an auxiliary substrate renders it a promising platform for the expression of additional heterologous enzymes that enable methanol to be used as an additional feedstock. Recently, we demonstrated that the *H. polymorpha* gene encoding alcohol oxidase could be functionally expressed in *S. cerevisiae* (31). We are currently exploring this and other options to achieve efficient cointilization of methanol by *S. cerevisiae*.

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